PHENOTYPE MODIFYING GENETIC SEQUENCES

The present invention relates generally to nucleic acid molecules capable of modifying phenotypic traits in eukaryotic cells and in particular plant cells. The nucleic acid molecules of the present invention are referred to as "phenotype modifying genetic sequences" or "PMGSs" and may be used to increase and/or stabilise or otherwise facilitate expression of nucleotide sequences being expressed into a translation product or may be used to down regulate by, for example, promoting transcript degradation *via* mechanisms such as co-suppression. The PMGSs of the present invention are also useful in modulating plant physiological processes such as but not limited to resistance to plant pathogens, senescence, cell growth, expansion and/or divsion and the shape of cells, tissues and organs. One particularly useful group of PMGSs modulate starch metabolism and/or cell growth or expansion or division. Another useful group of PMGSs are involved in increasing and/or stabilising or otherwise facilitating expression of nucleotide sequences in eukaryotic cells such as plant cells and in particular the expression of therapeutically, agriculturally and economically important transgenes. The PMGSs may also be used to inhibit, reduce or otherwise down regulate expression of a nucleotide sequence such as a eukaryotic gene, including a pathogen gene, the expression of which, results in an undesired phenotype. The PMGSs of the present invention generally result, therefore, in the acquisition of a phenotypic trait or loss of a phenotypic trait.

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

The subject specification contains nucleotide and amino acid sequence information prepared using the programme PatentIn Version 2.0, presented herein after the bibliography. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc). The length, type of sequence (DNA, protein (PRT), etc) and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by the information provided in numeric indicator field <400> followed by the

sequence identifier (eg. <400>1, <400>2, etc).

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Recombinant DNA technology is now an integral part of strategies to generate genetically modified eukaryotic cells. For example, genetic engineering has been used to develop varieties of plants with commercially useful traits and to produce mammalian cells which express a therapeutically useful gene or to suppress expression of an unwanted gene. Transposons have played an important part in the genetic engineering of plant cells and some non-plant cells to provide *inter alia* tagged regions of genomes to facilitate the isolation of genes by recombinant DNA techniques as well as to identify important regions in plant genomes responsible for certain physiological processes.

The maize transposon *Activator (Ac)* and its derivative *Dissociation (Ds)* was one of the first transposon systems to be discovered (1,2) and was used by Fedoroff *et al* (3) to clone genes. The behaviour of *Ac* in maize has been studied extensively and excision occurs in both somatic and germline tissue. Studies have highlighted two important features of *Ac/Ds* for tagging. First, the transposition frequency and second, the preference of *Ac/Ds* for transposition into linked sites.

The use of the *Ac/Ds* system has been hampered by the difficulty of data interpretation. One reason for this is the high activity of *Ac* in certain plants causing insertions at unlinked sites due to multiple transpositions, rather than a single event, from the T-DNA. This problem was addressed by Jones *et al* (4), Carroll *et al* (5) and others, and a two component *Ac/Ds* system was developed. In this system, *Ds* elements were made wherein the *Ac* transposase gene was replaced with a marker gene thereby rendering it non-autonomous. Separate *Ac* elements were then made. Subsequently, T-DNA regions of binary vectors carrying either a *Ds* element or a stabilised Activator transposase gene *(sAc)* were constructed by Carroll *et al* (5) and Scofield

et al (6).

The *Ds* element contained a reporter gene (eg. *nos:BAR)* which was shown to be inactivated on crossing with plants carrying the *sAc* (5). This is referred to as transgene silencing. It has been shown that transgene silencing is a more general phenomenon in transgenic plants (7, 8, 9). Many different types of transgene silencing have now been reported in the literature and include: co-suppression of a transgene and a homologous endogenous plant gene (10), inactivation of ectopically located homologous transgenes in transgenic plants (7), the silencing of transgenes leading to resistance to virus infection (11) and inactivation of transgenes inserted in maize transposons in transgenic tomato (5).

Gene silencing undoubtedly reflects mechanisms of great importance in the understanding of plant gene regulation. It is of particular importance because it represents a severe obstacle to stable and high level expression of economically important transgenes (7).

In work leading up to the present invention, the inventors sought to identify regulatory mechanisms involved in controlling expression of phenotypic traits in eukaryotic cells and in particular plant cells including modulating plant physiological processes, preventing or otherwise reducing gene silencing and/or facilitating increased and/or stabilized gene expression in eukaryotic cells such as plant cells. In accordance with the present invention, the subject inventors have identified and isolated phenotype modifying genetic sequences referred to herein as "PMGSs" which are useful in modifying expression of nucleotide sequences in eukaryotic cells such as plant cells.

One aspect of the present invention is predicated in part on the elucidation of the molecular basis of transposase-mediated silencing of genetic material located within a transposable element. Although, in accordance with the present invention, the molecular basis of gene silencing has been determined with respect to plant selectable marker genes within the *Ds* element of the *Ds/Ac* maize transposon system, the present invention clearly extends to the silencing of any nucleotide sequence and in particular a transgene and to mechanisms for alleviating gene silencing. In accordance with the present invention, nucleotide sequences have been identified

which alleviate gene silencing and which increase or stabilise expression of genetic material. Furthermore although the present invention is particularly exemplified in relation to plants, it extends to all eukaryotic cells such as cells from mammals, insects, yeasts, reptiles and birds.

Accordingly, an aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides which increases or stabilizes expression of a second nucleotide sequence inserted proximal to said first mentioned nucleotide sequence.

The term "proximal" is used in its most general sense to include the position of the second nucleotide sequence near, close to or in the genetic vicinity of the first mentioned nucleotide sequence. More particularly, the term "proximal" is taken herein to mean that the second nucleotide sequence precedes, follows or is flanked by the first mentioned nucleotide sequence. Preferably, the second nucleotide sequence is within the first mentioned nucleotide sequence and, hence, is flanked by portions of the first nucleotide sequence. Generally, the second nucleotide sequence is flanked by up to about 10 kb either side of first mentioned nucleotide sequence, more preferably up to about 5 kb, even more preferably up to about 1 kb either side of said first mentioned nucleotide sequence and even more preferably up to about 10 bp to about 1 kb.

Another aspect of the present invention is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides which stabilises, increases or enhances expression of a second nucleotide sequence inserted into, flanked by, adjacent to or otherwise proximal to the said first mentioned nucleotide sequence.

The second mentioned nucleotide sequence is preferably an exogenous nucleotide sequence meaning that it is either not normally indigenous to the genome of the recipient cell or has been isolated from a cell's genome and then re-introduced into cells of the same plant or animal, same species of plant or animal or a different plant or animal. More preferably, the exogenous sequence is a transgene or a derivative thereof which includes parts, portions, fragments and homologues of the gene.

The first mentioned nucleotide sequence described above is referred to herein as a "phenotype

modulating genetic sequence" or "PMGS" since it functions to and is capable of increasing or stabilizing expression of an exogenous nucleotide sequence such as a transgene or its derivatives. This in turn may have the effect of alleviating silencing of an exogenous nucleotide sequence or may promote transcript degradation such as *via* co-suppression. The latter is particularly useful as a defence mechanism against pathogens such as but not limited to plant viruses and animal pathogens.

Accordingly, another aspect of the present invention relates to a PMGS comprising a sequence of nucleotides which increases, enhances or stabilizes expression of a second nucleotide sequence inserted within, adjacent to or otherwise proximal to said PMGS.

PMGSs may or may not be closely related at the nucleotide sequence level although they are closely functionally related in modulating phenotypic expression. Particularly preferred PMGSs are represented in <400>1; <400>2; <400>3; <400>4; <400>5; <400>6; <400>7; <400>8; <400>9; <400>10; <400>11; <400>12; <400>13; <400>14; <400>15; <400>16; <400>17; <400>18; <400>19; <400>20; <400>21; <400>22; <400>23; <400>24; <400>25; <400>26; <400>27; <400>28; <400>29; <400>30 and/or <400>31 as well as nucleotide sequences having at least about 25% similarity to any one of these sequences after optimal alignment with another sequence of a sequence capable of hybridizing to any one of these sequences under low stringency conditions at 42°C.

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particularly preferred embodiment, nucleotide and sequence comparisons are made at the level of identity rather than similarity. Any number of programs are available to compare nucleotide and amino acid sequences. Preferred programs have regard to an appropriate alignment. One such program is Gap which considers all possible alignment and gap positions

and creates an alignment with the largest number of matched bases and the fewest gaps. Gap uses the alignment method of Needleman and Wunsch (24). Gap reads a scoring matrix that contains values for every possible GCG symbol match. GAP is available on ANGIS (Australian National Genomic Information Service) at website http://mel1.angis.org.au. Another particularly useful programme is "tBLASTx" (25).

Reference herein to a low stringency at 42°C includes and encompasses from at least about 0% v/v to at least about 15% v/v formamide and from at least about 1M to at least about 2M salt for hybridisation, and at least about 1M to at least about 2M salt for washing conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5M to at least about 0.9M salt for hybridisation, and at least about 0.5M to at least about 0.9M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01M to at least about 0.15M salt for hybridisation, and at least about 0.01M to at least about 0.15M salt for washing conditions.

Accordingly, another aspect of the present invention provides a PMGS comprising the nucleotide sequence:

<400>1; <400>2; <400>3; <400>4; <400>5; <400>6; <400>7; <400>8; <400>9; <400>10; <400>11; <400>12; <400>13; <400>14; <400>15; <400>16; <400>17; <400>18; <400>19; <400>20; <400>21; <400>22; <400>23; <400>24; <400>25; <400>26; <400>27; <400>28; <400>29; <400>30 and/or <400>31; or a sequence having at least 25% similarity after optimal alignment of said sequence to any one of the above sequences or a sequence capable of hybridizing to any one of the above sequences under low stringency conditions at 42°C.

Alternative percentage similarities or identities include at least about 30%, 40%, 50%, 60%, 70%, 80%, 90% or above.

A further aspect of the present invention is predicated on transposon-mediated tagging of tomato

plants which was shown to result in the identification of mutants exhibiting altered physiological properties. In particular, the insertion of a transposon in close proximity to the α-amylase gene resulted in continued or modified expression of the α-amylase gene past the initial development stage of the plant. In wild-type plants, negative regulatory mechanisms which may include methylation result in the non-expression of the α-amylase gene. In accordance with this aspect of the present invention, modified expression of the α-amylase gene, post or after initial developmental stage, results in physiological attributes such as altered senescence, altered resistance to pathogens, modification of the shape of plant cells, tissues and organs and altered cell growth or expansion or division characteristics. It is proposed, in accordance with the present invention, that the altered physiological phenotype is due to modified starch metabolism by the continued or modified expression of the α-amylase gene. In particular, increased or modified expression of the α-amylase gene or otherwise continued or altered expression of the α-amylase gene post initial development results in cell death, i.e. cell apoptosis, but also induces or promotes resistance to pathogens.

Accordingly, another aspect of the present invention contemplates a method for controlling physiological processes in a plant said method comprising modulating starch metabolism in cells of said plant.

More particularly, the present invention is directed to a method of inducing a physiological response in a plant said method comprising inhibiting or facilitating starch metabolism in cells of said plant after the initial developmental stage.

This aspect of the present invention is exemplified herein with respect to the effects of starch metabolism in tomato plants. This is done, however, with the understanding that the present invention extends to the manipulation of starch metabolism in any plant such as flowering plants, crop plants, ornamental plants, vegetable plants, native Australian plants as well as Australian and non-Australian trees, shrubs and bushes. The preferred means of modulating physiological process is *via* the introduction of a PMGS. In this context, a nucleotide sequence encoding an α-amylase gene or a portion or derivative thereof or a complementary sequence thereto, for example, would be regarded as a PMGS, as would a nucleotide sequence which promotes

increased and/or stabilised expression of a target gene.

The term "expression" is conveniently determined in terms of desired phenotype. Accordingly, the expression of a nucleotide sequence may be determined by a measurable phenotypic change involving transcription and translation into a proteinaceous product which in turn has a phenotypic effect or at least contributes to a phenotypic effect. Alternatively, expression may involve induction or promotion of transcript degradation such as during co-suppression resulting in inhibition, reduction or otherwise down-regulation of translatable product of a gene. In the latter case, the nucleic acid molecules of the present invention may result in production of sufficient transcript to induce or promote transcript degradation. This is particularly useful if a target endogenous gene is to be silenced or if the target sequence is from a pathogen such as a virus, bacterium, fungus or protozoan. In all instances "expression" is modulated but the result is conveniently measured as a phenotypic change resulting from increased or stabilised production of transcript thereby resulting in increased or stabilised translation product, or increased or enhanced transcript production resulting in transcript degradation leading to loss of translation product (such as in co-suppression).

The term "modulating" is used to emphasise that although transcription may be increased or stabilised, this may have the effect of either permitting stabilised or enhanced translation of a product or inducing transcription degradation such as *via* co-suppression.

Physiological responses and other phenotypic changes contemplated by the present invention include but are not limited to transgene expression, cell apoptosis, senescence, pathogen resistance, cell, tissue and organ shape and plant growth as well as cell growth, expansion and/or division.

In a particularly preferred embodiment, starch metabolism is stimulated, promoted or otherwise enhanced or inhibited by manipulating levels of an amylase and this in turn may lead to *inter alia* senescence or apoptosis as well as resistance to pathogens. Reference to "amylase" includes any amylase associated with starch metabolism including α-amylase and β-amylase. This aspect of the present invention also includes mutant amylases. In addition, the manipulation of levels of

amylase may be by modulating endogenous levels of a target plant's own amylase, or an exogenous amylase gene or antisense, co-suppression or ribozyme construct may be introduced into a plant. The exogenous amylase gene may be from another species or variety of plant or from the same species or variety or from the same plant. The present invention extends to recombinant amylases and derivative amylases including fusion molecules, hybrid molecules and amylases with altered substrate specifications and/or altered regulation. Any molecule capable of acting as above including encoding an α-amylase is encompassed by the term "PMGS".

According to another aspect of the present invention there is provided a method of inducing a physiological response in a plant such as but not limited to inducing resistance to a plant pathogen, enhancing or delaying senescence, modifying cell growth or expansion or division or altering the shape of cells, tissues or organs, said method comprising modulating synthesis of an amylase or functional derivative thereof for a time and under conditions sufficient for starch metabolism to be modified.

Preferably, the amylase is α-amylase.

The manipulation of amylase levels may also be by manipulating the promoter for the amylase gene. Again, the introduction of a PMGS may achieve such manipulation. Alternatively, an exogenous amylase gene may be introduced or an exogenous promoter designed to enhance expression of the endogenous amylase gene. A PMGS extends to such exogenous amylase genes and promoters.

One group of PMGSs of the present invention were identified following transposon mutagenesis of plants with the *Ds/Ac* transposon system. The *Ds* element carries a reporter gene *(nos:BAR)* which is normally silenced upon exposure to the transposase gene. In a few cases, plants are detected in which *nos:BAR* expression is not silenced. In accordance with the present invention, it has been determined that the *Ds* element inserts within, adjacent to or otherwise proximal with a PMGS which results in increased or stabilized expression of the *nos:BAR*. In other words, the PMGS facilitates expression of a gene and preferably an exogenous gene or a transgene. This in turn may result in a gene product being produced or induction of transcript degradation such

as *via* co-suppression.

The PMGSs of the present invention are conveniently provided in a genetic construct.

Accordingly, another aspect of the present invention contemplates a genetic construct comprising a PMGS as herein defined and means to facilitate insertion of a nucleotide sequence within, adjacent to or otherwise proximal with said PMGS.

The term "genetic construct" is used in its broadest sense to include any recombinant nucleic acid molecule and includes a vector, binary vector, recombinant virus and gene construct.

The means to facilitate insertion of a nucleotide sequence include but are not limited to one or more restriction endonuclease sites, homologous recombination, transposon insertion, random insertion and primer and site-directed insertion mutagenesis. Preferably, however, the means is one or more restriction endonuclease sites. In the case of the latter, the nucleic acid molecule is cleaved and another nucleotide sequence ligated into the cleaved nucleic acid molecule.

Preferably, the inserted nucleotide sequence is operably linked to a promoter in the genetic construct.

According to this embodiment, there is provided a genetic construct comprising an PMGS as herein defined and means to facilitate insertion of a nucleotide sequence within, adjacent to or otherwise proximal with said PMGS and operably linked to a promoter.

Conveniently, the genetic construct may include or comprise a transposable element such as but not limited to a modified form of a *Ds* element. A modified form of a *Ds* element includes a *Ds* portion comprising a PMGS and a nucleotide sequence such as but not limited to a reporter gene, a gene conferring a particular trait on a plant cell or a plant regenerated from said cell or a gene which will promote co-suppression of an endogenous gene.

Another aspect of the present invention contemplates a method of increasing or stabilising

expression of a nucleotide sequence or otherwise preventing or reducing silencing of a nucleotide sequence or promoting transcription degradation of an endogenous gene in a plant or animal or cells of a plant or animal, said method comprising introducing into said plant or animal or plant or animal cells said nucleotide sequence flanked by, adjacent to or otherwise proximal with a PMGS.

In an alternative embodiment, there is provided a method of inhibiting, reducing or otherwise down-regulating expression of a nucleotide sequence in a plant or animal or cells of a plant or animal, said method comprising introducing into said plant or animal or plant or animal cells the nucleotide sequence flanked by, adjacent to or otherwise proximal with a PMGS.

Yet another aspect of the present invention provides a transgenic plant or animal carrying a nucleotide sequence flanked by, adjacent to or otherwise proximal to a PMGS. As a consequence of the PMGS, the expression of the exogenous nucleotide sequence is increased or stabilised resulting in expression of a phenotype or loss of a phenotype.

Although not intending to limit the present invention to any one theory or mode of action, one group of PMGSs is proposed to comprise a methylation resistance sequence. A methylation resistance sequence is one which may de-methylate and/or prevent or reduce methylation of a nucleotide sequence such as a target nucleotide sequence.

The present invention further extends to a transgenic plant or a genetically modified plant exhibiting one or more of the following characteristics:

(i) an amylase gene not developmentally silenced;
(ii) an amylase gene capable of constitutive or inducible expression;
(iii) a mutation preventing silencing of an amylase gene;
(iv) a nucleic acid molecule proximal to an amylase gene and which substantially prevents methylation of said amylase gene;
(v) decreased amylase gene expression; and/or
(vi) a genetically modified amylase allele(s).

Reference herein to a "gene" is to be taken in its broadest context and includes:

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5′- and 3′-untranslated sequences)'

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) optionally comprising 5′- or 3′-untranslated sequences of the gene; or

(iii) an amplified DNA fragment or other recombinant nucleic acid molecule produced *in vitro* and comprising all or a part of the coding region and/or 5′- or 3′- untranslated sequences of the gene.

The term "proximal" is used in its most general sense to include the position of the amylase gene near, close to or in the genetic vicinity of the nucleic acid molecule referred to in part (iv) above. More particularly, the term "proximal" is taken herein to mean that the amylase gene precedes, follows or is flanked by the nucleic acid molecule. Preferably, the amylase is within the nucleic acid molecule and, hence, is flanked by portions of the nucleic acid molecule. Generally, the amylase gene is flanked by up to about 100 kb either side of the nucleic acid molecule, more preferably up to about 10 kb, even more preferably to about 1 kb either side of the nucleic acid molecule and even more preferably up to about 10 bp to about 1 kb.

Accordingly, another aspect of the present invention is directed to a PMGS comprising a sequence of nucleotides which stabilises, increases or enhances expression of an amylase gene inserted into, flanked by, adjacent to or otherwise proximal to the said nucleic acid molecule.

In an alternative embodiment, the present invention contemplates a PMGS comprising a sequence of nucleotides which inhibits, decreases or otherwise reduces expression of an amylase gene inserted into, flanked by, adjacent to or otherwise proximal to the said nucleic acid molecule.

The term "expression" is conveniently determined in terms of desired phenotype. Accordingly, the expression of a nucleotide sequence may be determined by a measurable phenotypic change such as resistance to a plant pathogen, enhanced or delayed senescence, altered cell growth or

expansion or division or altered cell, tissue or organ shape.

The PMGS of this aspect of the present invention functions to and is capable of modulating expression of an amylase gene or its derivatives. The term "modulating" includes increasing or stabilising expression of the amylase gene or decreasing or inhibiting the amylase gene. The PMGS may be a co-suppression molecule, ribozyme, antisense molecule, an anti-methylation sequence, a methylation-inducing sequence and/or a negative regulatory sequence, amongst other molecules.

Accordingly, another aspect of the present invention relates to a PMGS comprising a sequence of nucleotides which increases, enhances or stabilizes expression of an amylase gene inserted within, adjacent to or otherwise proximal with said PMGS.

In an alternative embodiment, the present invention provides a PMGS comprising a sequence of nucleotides which inhibits, decreases or otherwise reduces expression of an amylase gene inserted within, adjacent to or otherwise proximal with said PMGS.

Another aspect of the present invention contemplates a genetic construct comprising a PMGS as herein defined and means to facilitate insertion of a nucleotide sequence within, adjacent to or otherwise proximal with said PMGS wherein said nucleotide sequence encodes an amylase or functional derivative thereof.

Preferably, the amylase gene sequence is operably linked to a promoter in the genetic construct.

According to this embodiment, there is provided a genetic construct comprising an PMGS as herein defined and means to facilitate insertion of a nucleotide sequence within, adjacent to or otherwise proximal with said PMGS and operably linked to a promoter wherein said nucleotide sequence encodes an amylase or functional derivative thereof.

Conveniently, the genetic construct may be a transposable element such as but not limited to a modified form of a *Ds* element. A modified form of a *Ds* element includes a *Ds* portion

comprising a PMGS and a nucleotide sequence such as but not limited to a reporter gene and a gene encoding an amylase.

Another aspect of the present invention contemplates a method of increasing or stabilising expression of a nucleotide sequence encoding an amylase or otherwise preventing or reducing silencing of a nucleotide sequence encoding an amylase in a plant cell said method comprising introducing into said plant or plant cells said nucleotide sequence encoding an amylase flanked by, adjacent to or otherwise proximal with a PMGS.

In an alternative embodiment, the present invention provides a method of inhibiting, decreasing or otherwise reducing expression of a nucleotide sequence encoding an amylase in a plant cell said method comprising introducing into said plant or plant cells said nucleotide sequence encoding an amylase flanked by, adjacent to or otherwise proximal with a PMGS.

Yet another aspect of the present invention provides a transgenic plant carrying a nucleotide sequence encoding an amylase flanked by, adjacent to or otherwise proximal with a PMGS.

Still a further aspect of the present invention provides nucleic acid molecules encoding apoptotic peptides, polypeptides or proteins or nucleic acid molecules which themselves confer apoptosis. One example of an apoptotic nucleic acid molecule is a molecule capable of inducing or enhancing amylase synthesis. Other molecules are readily identified, for example, by a differential assay. In this example, nucleic acid sequences (e.g. DNA, cDNA, mRNA) are isolated from wild type plants and mutant plants which exhibit enhanced or modified amylase gene expression. The differential assay seeks to identify DNA or mRNA molecules in the mutant plant or wild type plant which are absent in the respective wild type plant or mutant plant. Such nucleic acid molecules are deemed putative apoptosis-inducing or apoptosis-inhibiting genetic sequences. These molecules may have utility in regulating beneficial physiological processes in plants.

Another aspect of the present invention contemplates a method for controlling physiological processes in a plant said method comprising modulating cell shape and/or expansion and/or

division or growth in said plant.

More particularly, the present invention is directed to a method of inducing a physiological response in a plant said method comprising enhancing or facilitating the manipulation of cell shape and/or expansion or division or growth in said plant.

This aspect of the present invention is based on the detection of a *Ds* insertion in the *Dem* gene in plants. The *Dem* gene is highly expressed in shoot and root apices. The resulting mutation results in genetically-modified palisade tissue. Mutant lines exhibiting altered cell shape or expansion or division or growth are selected and, in turn, further lines exhibiting such beneficial characteristics as increased levels of photosynthetic activity are obtainable. The two basic processes which contribute to plant shape and form are cell division and cell expansion or growth. By somatically tagging *Dem*, the inventors have demonstrated that *Dem* is required for expansion or division or growth of palisade and adaxial epidermal cells during leaf morphogenesis. Therefore, the primary role of the DEM protein in plant morphogenesis in general is in cell expansion or division or growth rather than the orientation or promotion of cell division.

Accordingly, another aspect of the present invention provides a method of inducing a physiological response in a plant such as but not limited to inducing resistance to a plant pathogen, enhancing or delaying senescence, modifying cell growth or expansion or division or altering the shape of cells, tissues or organs, said method comprising modulating expression of the *Dem* gene.

Still yet another aspect of the present invention relates to a transgenic plant or a genetically modified plant exhibiting one or more of the following properties:

(i) a *Dem* gene not developmentally silenced;

(ii) a *Dem* gene capable of constitutive or inducible expression;

(iii) a mutation preventing silencing of the *Dem* gene;

(iv) a nucleic acid molecule proximal to the *Dem* gene and which substantially prevents

methylation of said *Dem* gene or demethylates the *Dem* gene;

(v) decreased *Dem* gene expression; and/or

(vi) a genetically modified *Dem* allele(s).

The present invention is further directed to the putative *Dem* promoter and its derivatives. The *Dem* promoter is approximately 700 bases in length extending upstream from the ATG start site. The nucleotide positions of putative *Dem* promoter are nucleotide 3388 to 4096 (Figure 5). The nucleotide sequence of the *Dem* promoter is set forth in <400>8.

Yet another aspect of the present invention is directed to a mutation in or altered expression of a putative patatin gene in tomato or other plants. The patatin gene is referred to herein as "putative" as it exhibits homology to the potato patatin gene.

Accordingly, another aspect of the present invention contemplates a method for controlling physiological processes in a plant said method comprising modulating C metabolism in cells of said plant.

More particularly, the present invention is directed to a method of inducing a physiological response in a plant said method comprising enhancing or facilitating C metabolism in cells of said plant.

Another aspect of the present invention provides a method of inducing a physiological response in a plant such as but not limited to inducing resistance to a plant pathogen, enhancing or delaying senescence, modifying cell growth or expansion or division or altering the shape of cells, tissues or organs, said method comprising modulating expression of a putative patatin gene or a functional derivative thereof.

Still yet another aspect of the present invention relates to a transgenic plant or a genetically modified plant exhibiting one or more of the following properties:

(i) a putative patatin gene not developmentally silenced;

(ii) a putative patatin gene capable of constitutive or inducible expression;

(iii) a mutation preventing silencing of a putative patatin gene;

(iv) a nucleic acid molecule proximal to a putative patatin gene and which substantially prevents methylation of said putative patatin gene or demethylates said putative patatin gene;

(v) decreased putative patatin gene expression; and/or

(vi) a genetically modified patatin allele(s).

Reference herein to "genetically modified" genes such as an altered amylase, *Dem* or patatin allele includes reference to altered plant development genes. The present invention is particularly directed to alteration of alleles which leads to economically physiologically or agriculturally beneficial traits.

The present invention further provides for an improved transposon tagging system.

One system employs a modified *Ds* element which now carries a PMGS.

Accordingly, another aspect of the present invention is directed to an improved transposon tagging system, said system comprising a transposable element carrying a nucleotide sequence flanked by, adjacent to or otherwise proximal with a PMGS.

Another new system employs the *Dem* gene or its derivatives as an excision marker. Reference to "derivatives" includes reference to mutants, parts, fragments and homologues of *Dem* including functional equivalents. The *Dem* gene is required for cotyledon development and shoot and root meristem function. Stable *Ds* insertion mutants of *Dem* germinate but fail to develop any further. However, unstable mutants in the *Dem* locus result in excision of the *Ds* element and reversion of the *Dem* locus to wild-type, thereby restoring function to the shoot meristem. In accordance with the present invention, the new system enables selection for transposition.

In accordance with the improved method, transposition is initiated by crossing a *Ds*-containing line with a stabilized *Ac (sAc)*-containing line. The *Ds*-containing line is heterozygous for a *Ds*

insertion in the *Dem* gene and the *sAc* line is heterozygous for a stable mutation in the *Dem* gene. A particularly useful mutant in the *Dem* gene is a stable frameshift mutation. Both of the *Ds*- and *sAc*- containing plant lines are wild-type due to the recessive nature of the *Ds* insertion and mutant alleles. The F_1 progeny derived from crossing the *Ds* and *sAc* lines segregate at a ratio of 3 wild-types to 1 mutant. Because the *sAc* is linked to the frameshift *dem* allele, almost all of the F_1 mutants also inherit the transposase gene and can undergo somatic reversion. These revertant individuals have abnormal cotyledons, but *Ds* excision from the *Dem* gene restores function to the shoot apical meristem. Each somatic revertant represents an independent transposition event from the *Dem* locus. By screening for expression of a gene resident on the *Ds* element (e.g. *nos:BAR),* the identification of PMGSs is readily determined.

The present invention also provides *in vivo* bioassays for expressed transgenes. The bioassays identify nucleotide sequences which prevent transgene silencing.

In one aspect, the plant expression vector pZorz carries a firefly luciferase reporter gene *(luc),* under the control of the *Osa* promoter (12). After bombardment, the gene is expressed in embryogenic sugarcane callus. However, it becomes completely silenced upon plant regeneration. The silencing appears to be correlated with methylation of the transgene. Genetic sequences flanking reactivated *nos:BAR* insertions are inserted into modified forms of the pZorz expression vector. These pZorz constructs are then used with a transformation marker to transform sugarcane in order to test whether the plant sequences are capable of alleviating silencing of the *luc* gene upon plant regeneration. Restriction endonuclease fragments capable of alleviating silencing of the *luc* gene are subject to deletion analysis and smaller fragments are subcloned into modified pZorz expression vectors to define the sequences more accurately (Figure 7).

In another aspect, a plant expression vector is constructed for testing the PMGSs in *Agrobacterium*-transformed *Arabidopsis.* PMGSs are placed upstream of the *nos:luc* or *nos:gus* gene linked to a transformation marker and used to test whether PMGS s stabilise expression of the *nos:luc* or *nos:gus* gene in *Arabidopsis.*

These aspects of the present invention are clearly extendable to assays using other plants and the present invention contemplates the subject assay and plant expression vector for use in a range of plants in addition to sugar cane.

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The present invention is further described by the following non-limiting Figures and Examples.

In the Figures:

Figure 1 is a diagrammatic representation showing T-DNA regions of binary vectors carrying a *Ds* element (SLJ1561) of the transposable gene (SLJ10512)[5]. The *Ds* element carries a *nos:BAR* gene and is inserted into a *nos:SPEC* excision marker. The transposon gene *sAc* is linked to a 2':*Gus* reporter gene.

Figure 2 is a diagrammatic representation showing an experimental strategy for generating tomato lines carrying transposed *Ds* elements (5). F1 plants heterozygous for both the *Ds* and *sAc* T-DNAs are test-crossed to produce TC_1 progeny. The TC_1 progeny are then screened for lines carrying a transposed *Ds* and a reactivated *nos:BAR* gene.

Figure 3 is a representation showing methylation of a genetically engineered *Ds* transposon in transgenic tomato. Two separate Southern analyses were conducted on 7 individual genotypes; genomic DNA was extracted from leaf tissue (5). The restriction enzymes and probes (shaded boxes) used are shown on the figure. Lanes: 1. Non transformed (i.e. no *Ds* or *nos:BAR* gene), 2. 1561E which carries an active *nos:BAR* gene (due to the fact that it has never been exposed to the transposase gene), 3-6. Four tomato lines that carry silent *nos:BAR* genes, 7. UQ406 which carries an active *nos:BAR* gene due to insertion of the *Ds* in the α-amylase promoter. The enzymes *Sst*II (abbreviated Ss) and *Not*I (abbreviated Nt) are methylation sensitive, whereas *Bst*YI (abbreviated Bs) and *Eco*RI (abbreviated RI) are not. The expected size fragment for unmethylated DNA is indicated by the arrow; larger fragments (as in the silent lines) indicate methylation of the DNA at the *Sst*II or *Not*I sites.

Figure 4 is a representation showing a sequence comparison between the potato α-amylase promoter (15) <400>2 and the tomato α-amylase promoter <400>1. The location of the UQ406 insertion is shown.

Figure 5 is a representation of a nucleotide sequence <400>3 of tomato genomic DNA from 651

bp upstream of the *Ds* insertion (acttcgag: underlined) in UQ406 to the beginning of the *Dem* coding sequence, followed by the *Dem* cDNA sequence from the ATG start site at base pair 4097 (sequence underlined). The target sequences of the *Ds* insertion in UQ406 and *Dem* ATG are underlined. The *Dem* cDNA sequence is shown in italics and underlined. The putative *Dem* promoter begins at nucleotide 3388 and ends just immediately prior to the ATG, i.e. at position 4096 <400>8.

Figure 6 is a diagrammatic representation showing an improved transposon tagging strategy using *Dem* as excision marker. The *sAc* and *Ds* parent lines are represented by the upper left and right boxes, respectively. Because the *sAc* is linked to the *dem* mutant +7 allele, somatic revertants can theoretically occur at about the frequency of 1 out of 4 in the F1 progeny. Each somatic revertant represents an independent transposition event. Chr4, chromosome 4 of tomato.

Figure 7 is a diagrammatic representation showing construction of pUQ expression vectors from the pZorz vector (12; see Example 9).

Figure 8 is a representation of somatic tagging of the *Dem* locus. **a**. Diagrammatic representation of the STD (somatic tagging of *Dem)* genotype. *dem*+7 is a stable frameshift mutant of *Dem,* TPase represents a T-DNA 3 centiMorgans (cM) from *Dem,* carrying the *Ac* transposase and a GUS reporter gene. The transposase is required for *Ds* transposition. **b**. Location of stably inherited (shaded) and somatic (open) *Ds* insertions in the *Dem* locus and an upstream α-amylase gene. The α-amylase gene is in the same orientation as *Dem.* Coding sequences plus introns are shown as boxes and the dark section of the *Dem* locus represents an intron. All of the 8 somatic insertions shown in the figure were associated with palisade deficient sectors. The genomic region represented in **b** has been sequenced (see Figure 5; please note that the intron in the *dem* locus is not included in this sequence). **c**. Mutant *dem* sectors lack palisade cells (p, palisade cells, s, spongy mesophyll, g, wild-type dark green sectors, and lg, mutant light green sectors).

Figure 9 shows PCR on intact tissue of *dem* sectors. **M**, 1 kb ladder. **1-10**, unique *Ds* insertions

in *Dem* detected by PCR. Intact leaf tissues (mutant somatic sectors) were used as template in the PCR. PCR with oligonucleotide primers facing out of *Ds* and in the *Dem* coding sequence amplified unique fragments from each mutant sector, thereby confirming that the sectors shown in Figure 8 are indeed mutant *dem* sectors.

Figure 10 is a diagrammatic representation of the genetic derivation of plants containing independent somatic *dem* alleles. Somatic revertants were generated by crossing plants heterozygous for the dem^{+7} mutant allele linked to transposase (sAc,GUS) and plants heterozygous for the dem^{Ds} mutant allele. Revertant seedlings were selfed and GUS^{+} individuals were identified. From 150 somatic revertants, four independent lines were produced carrying hundreds of independent *dem* alleles.

Figure 11 is a photographic representation showing a multicellular palisade mutant allele of the *Dem* locus. At the single-cell embryo stage, the plant possessing the multicellular palisade sector shown carried a transposase gene and was heterozygous for a mutant frameshift allele and a wild-type allele of the *Dem* locus. During development, however, mutant *dem* sectors were produced due to the insertion of a *Ds* element into the wild-type allele. Wild-type palisade tissue is essentially composed of single long columnar cells. Some mutant sectors (due to *Ds* insertion) totally lack palisade cells (refer to the figure), whereas other mutant sectors have multicellular palisade tissue composed of small, non-columnar cells.

Figure 12 is a representation of the nucleotide sequence upstream of the UQ11 *Ds* insertion. The UQ11 *Ds* insertion resulted from transposition of the *Ds* back into the T-DNA. Nucleotide 1 is the first nucleotide upstream of *Ds* (containing an active *nos:BAR* gene). Nucleotide 1 to 295 correspond to *Agrobacterium* sequence from the right border of tomato transformant 1561E (5), the starting position of the *Ds* before loding in the *Dem* locus. Nucleotides 296 to 886 (in italics) correspond to tomato genomic DNA flanking the T-DNA insertion in 1561E. Note the *Bam*HI/*Bcl*I fusion sequence (TGACTC) and the *Hpa*I site (GTTAAC), both underlined in the figure immediately upstream of the insertion site. The putative PMGSs of UQ11 reside in the right border of the T-DNA (nucletoide 1 to 295), and/or the flanking tomato DNA (nucleotide 296 to 886), or further upstream.

Figure 13 is a diagrammatic representation of the T-DNA construct SLJ 1561 used to transform tomato to produce 1561E(5), and the location of the *Ds* element in UQ11. The *Ds* element in UQ11 is slightly closer to the right border (RB) and in the opposite orientation compared to the *Ds* element in 1561E.

TABLE 1

SUMMARY OF SEQUENCE (SEQ) IDENTIFIERS

SEQ IDENTIFIER	DESCRIPTION
<400>1	Nucleotide sequence of tomato α-amylase gene promoter
<400>2	Nucleotide sequence of potato α-amylase gene promoter
<400>3	Nucleotide sequence of genomic DNA upstream of *Dem* gene followed by *Dem* cDNA coding sequence in tomato line UQ406
<400>4	Nucleotide sequence upstream of *Ds* insertion (ie. upstream of the *nos:BAR* gene) in a putative patatin gene in tomato line UQ12
<400>5	Nucleotide sequence downstream of *Ds* insertion (ie. downstream of the *nos:BAR* gene) in a putative patatin gene in tomato line UQ12
<400>6	Nucleotide sequence of portion of putative tomato (UQ12) homologue of potato patatin gene
<400>7	Nucleotide sequence of portion of potato patatin gene having homology to <400>6
<400>8	Nucleotide sequence of putative *Dem* promoter in UQ406
<400>9	Nucleotide sequence upstream of *Ds* insertion in tomato mutant UQ11
<400>10	Putative PMGS from UQ11 corresponding to nucleotides 1 to 295 of <400>9
<400>11	Putative PMGS from UQ11 corresponding to nucleotide 296 to 836 of <400>9

<400>12	Nucleotide sequence of an upstream portion of putative sucrose synthase gene in tomato (UQ14) containing PMGS
<400>13	Nucleotide sequence of an downstream portion of putative sucrose synthase gene in tomato (UQ14) containing PMGS
<400>14	Putative PMGS from UQ14
<400>15	Partial nucleotide sequence of 3′ untranslated region from potato sucrose synthase
<400>16	PMGS from UQ14
<400>17	Partial nucleotide sequence of 3′ untranslated region from potato sucrose synthase
<400>18	PMGS from UQ14
<400>19	Partial nucleotide sequence of 3′ untranslated region from potato lactate dehydrogenase (LDH)
<400>20	PMGS from UQ14
<400>21	Partial nucleotide sequence of intron II of tomato phytochrome B1 (PHYB1)
<400>22	PMGS from UQ14
<400>23	Partial nucleotide sequence of 3′ untranslated region from potato sucrose synthase
<400>24	PMGS from UQ14
<400>25	Partial nucleotide sequence of 3′ untranslated region of potato lactate dehydrogenase (LDH)
<400>26	PMGS from UQ14
<400>27	Partial nucleotide sequence of intron I of potato cytosolic pyruvate kinase (CPK)
<400>28	PMGS from UQ14

<400>29	Partial nucletoide sequence downstream of *Brassica napus* 1.7S seed storage protein, napin *(napA)*
<400>30	PMGS from UQ14
<400>31	Partial nucleotide sequence of 3′ untranslated region of tomato chorismate synthase 2 precursor gene (CSP)
<400>32	Nucleotide sequence of an upstream portion of *Ds* insert containing PMGS in tomato (line UQ13)
<400>33	Nucleotide sequence of an downstream portion of *Ds* insert containing PMGS in tomato (line UQ13)
<400>34	PMGS from UQ13
<400>35	Partial nucleotide sequence of tomato expansin 2
<400>36	PMGS from UQ13
<400>37	Partial nucleotide sequence of tomato ADP-glucose pyrophosphorylase
<400>38	PMGS from UQ12
<400>39	Partial nucleotide sequence of tomato Ca^{2+} ATPase

EXAMPLE 1

Ds/sAc Transposon system

The inventors have previously developed a two component *Ds/sAc* transposon system in transgenic tomato for tagging and cloning important genes from plants (5, 13). The components of the system are shown in Figure 1 and comprise: i) a non-autonomous genetically-engineered *Ds* element (e.g. SLJ1561), and ii) an unlinked transposase gene *sAc* (SLJ10512), required for transposition of the *Ds* element. To activate transposition, the two components are combined by crossing transformants for each component. A plant selectable marker gene, e.g. *nos:BAR*, is inserted into the *Ds* element to enable selection for reinsertion of the elements following excision from the T-DNA (Figure 1). The marker gene is irreversibly inactivated when the *Ds* line is crossed to a transformant expressing the transposase gene (5). Silencing occurred when the *Ds* element remained in its original position in the T-DNA, and also occurred in the great majority of cases when the *Ds* element transposed to a new location in the tomato genome. The silenced marker gene has been shown to be stably inherited, even after the transposase gene segregates away from the *Ds* element in subsequent generations.

EXAMPLE 2

Transposon tagging of a chromosomal region enabling full expression of the *nos:BAR* transgene

The experimental strategy for generating tomato lines carrying transposed *Ds* elements from T-DNA 1561E is shown in Figure 2. The *Ds* element in 1561E carries a *nos:BAR* marker gene. In construction of the *Ds*, the 5' end of the *nos* promoter is cloned into the *Xho* I site, 1100 bp from the 3′ end of *Ac*. Hundreds of plants carrying transposed *Ds* elements are screened for resistance to phosphinothricin (PPT), the selection agent for the *BAR* gene. Surprisingly, several lines are identified which show at least some level of resistance. One line, called UQ406, carries a single transposed *Ds* element (without the transposase gene which has segregated away) and is resistant to PPT. Stable inheritance of *BAR* gene expression in this line has been demonstrated through several generations. These results indicate that the strategy for tagging active chromosomal regions by screening for PPT resistance is a successful approach.

Southern hybridization analysis of the original *Ds* transformant 1561E, UQ406 and several lines carrying silenced *nos:BAR* transgenes indicates that silencing is correlated with methylation of the *Sst*II site in the *nos* promoter (Figure 3). Total leaf tissue is used in this analysis, and the *Sst*II site in the *nos* promoter in UQ406 is only partially methylated, enabling sufficient expression of the *bar* gene to confer resistance. In silent *nos:BAR* genes, the *Sst*II site and *Not*I site immediately downstream from the coding sequence are both methylated (Figure 3). In UQ406, the *Not*I site is unmethylated, as in 1561E (Figure 3).

EXAMPLE 3

Cloning sequences flanking an active *nos:BAR* gene

GenomeWalker (14) is used to clone the tomato DNA sequences flanking the *Ds* element in UQ406. The DNA flanking the *Ds* element in line UQ406 is cloned and sequenced, and a search of the PROSITE database reveals that the *Ds* has inserted into the promoter region of an α-amylase gene. The promoter <400>1 shows strong similarity to an α-amylase promoter of potato (15; Figure 4) <400>2 and the coding sequence of the gene has strong homology with one of 3 reported potato α-amylase cDNAs (16). The DNA from 651 bp upstream of the UQ406 insertion to the end of the *Dem* coding sequence, has been sequenced (Figure 5) <400>3. Other such sequences have been located and cloned (see below) using the method of Example 4. Nucleotide sequences disclosed herein which flank the active *nos:BAR* gene are designated "phenotype modulating genetic sequences" or "PMGSs".

EXAMPLE 4

An improved transposon tagging strategy for transgenic tomato

The inventors have used the transposon tagging system described in Example 1 (also see Figure 2) to tag and clone two important genes involved in shoot morphogenesis. The *DCL* gene is required for chloroplast development and palisade cell morphogenesis (13) and the *Dem* (*D*efective *E*mbryo and *M*eri stem) gene is required for cotyledon development and shoot and root meristem function. Stable *Ds* insertion mutants of *Dem* germinate but fail to develop any further. In contrast, the unstable *Dem* seedlings appear at first to be mutant but the transposase

gene activates transposition of the *Ds* and reversion of the *Dem* locus to wild-type, thereby restoring function to the shoot meristem.

While the transposon tagging system described in Figure 2 has been successful in tagging genes and a chromosomal region alleviating transgene silencing, it does have two associated inefficiencies. First, transposition cannot be selected in the shoot meristem of F_1 plants heterozygous for *Ds* and *sAc*. As a consequence, many TC_1 progeny derived from test-crossing these F_1 plants still have the *Ds* located in the T-DNA. The other limitation of the system is that sibling TC_1 progeny derived from a single F_1 plant often carry the same clonal transposition and reinsertion event. The extent of clonal events amongst sibling TC_1 progeny can only be monitored by time consuming and expensive Southern hybridisation analysis.

These two inefficiencies in the transposon tagging strategy are overcome in accordance with the present invention by using the *Dem* gene as an excision marker. The new system enables selection for transposition in the shoot apical meristem and visual identification of plants carrying independent transposition events. Transposition is initiated by crossing a *Ds* line with a *sAc* line (Figure 6): The *Ds* line is heterozygous for a *Ds* insertion in the *Dem* gene and the *sAc* line is heterozygous for a stable frameshift mutation in the *Dem* gene (Figure 6). The frameshift allele is derived from a *Ds* excision event from the *Dem* locus. Both the *Ds* and *sAc* lines are wild-type due to the recessive nature of the *Ds* insertion and frameshift alleles. PCR tests on intact leaf tissue have been developed for the rapid identification of these *Ds* and *sAc* parental lines. The F_1 progeny derived from crossing the *Ds* and *sAc* lines segregate at the expected ratio of 3 wild-types to 1 mutant. Because the *sAc* is linked to the frameshift *dem* allele, almost all of the F_1 mutants also inherit the transposase gene *(sAc)* and can undergo somatic reversion. These revertant individuals have abnormal cotyledons, but *Ds* excision from the *Dem* gene restores function to the shoot apical meristem. Each somatic revertant represents an independent transposition event from the *Dem* locus. A non-destructive test for *nos:BAR* expression is used involving application of phosphinothricine [PPT] (the selective agent for expression of *BAR* gene) to a small area of a leaf. Somatic revertants resistant to PPT are grown though to seed and the F_2 progeny are screened again for PPT resistance. Lines carrying transposed *Ds* elements expressing *nos:BAR* are selected for more detailed molecular analysis. Four additional

independent insertions carry active *nos:BAR* genes. These mutants are UQ11, UQ12, UQ13 and UQ14. The donor *Ds* was originally located in the *Dem* gene (Figure 3) and in that location in the *Dem* gene the *nos:BAR* gene was silent. These independent lines were selected for further analysis (see Examples 5 and 6).

The efficient saturation mutagenesis of this chromosomal region is dependent on the use of the *Dem* gene as a selectable marker for independent transposition events. A recombinant selectable marker for independent transpositions is produced and transformed into tomato for saturation mutagenesis in other chromosomal regions of tomato. This system may be introduced into any species possessing the *dem* mutation, in order to facilitate transposon tagging of genes.

EXAMPLE 5

Ds transposon tagging of a putative patatin gene

DNA sequences flanking the active *nos:BAR* in a line designated UQ12 have similarly been cloned and sequenced. The flanking DNA appears to correspond to an intron in a homologous potato patatin gene. Patatin is the major protein in the potato tuber and has many potentially-important characteristics. For example, it possesses antioxidant activity; it has esterase activity and is potentially a phospholipase or lipid acylhydrolase (hydrolyzing phospholipase, liberating free fatty acids); it is induced during disease resistance; and it inhibits insect larval growth.

The sequence upstream of the *Ds* insertion (i.e. upstream of the *nos:BAR* gene) is as follows:

```
AATCAAAGAG GAATTNAATT CCNCAAAATT TCATCCATAG ATTTTGNGTC      50
TCTGAAAATT AAAGTGACTT TGTAATCTGA AACCTAGAGT CCTCAACCAT     100
ATCATTGACC ATTAAGCCAT ACCCTTAAAT GTAGGGAATT TGAAGTTTTA     150
AAAACCACAC TTTGTTATTT ATTGGCCCAA ATACTCGATA ATCTTTACAT     200
TATTGAAAAT CAACATTCAA AAGGAACGAA CCTTCAATCA CACCATCAAT     250
GTCAACTTTC TTTTATTTTG GATAATCTAA GTTTTTAAAT TGCAGTAAAA     300
TNAAATAAAA CCCTAAACTT CTTCTAGGTT GAGACTTAGT AAATATGAAT     350
TATATAAAGA ATTCATGACA AATGAGACAT AAGAATAGTG CCAGCAAATT     400
ACTTTTTTGA TATCTTATCT GTGATATCGG AATTTTAACT ACCATAAATT     450
TATGAATGAA ATATCACTTA TCTATTAGAG AGGATTTAAT CTCCCTTATA     500
ATGACATTGA TAAAAGCAAG NACAAGTGCT CTTTATTTCT TAATTACAAA     550
TCCTTAAATA GATAAAAGCT ACGAATAACA TAATATCCTT AAATAGATAA     600
```

```
AAGCTACGAA TAACATAATA GTATATTACT CCNAATTATT TTGATTTATT          650
TAAAATGACT CCACTAATCC TGATGTGGTC TAGG <400>4                    684
```

The tomato sequence immediately downstream of the *Ds* insertion (i.e. downstream of the *nos:BAR* gene) is as follows:

```
GGTCTAGGCC CTGGGTCTAG GAAACAAAAT AACTTATTTG ACTCCTAAAC           50
AATAGCAACA TACAAACCAC TGATATTGTA CAAGTAAAAT TCAATAAAAT          100
TCTAGCTCTC TCAAACACTT TTAAAATTGT TATTTCTGTT TTGTCTGTGT          150
CATATTATGA CCTACACAAC AACAACAACA ACGAATTTAG TGAAACTCTA          200
CAAAGTGGAG CCTGAAGTCG AGAGTTTACG CGGGCCTTAT CACTATCTTT          250
TCGAGATAAA AAAATTATTT TTAAAAGATC ATCGACTTAA ACAAACCAAA          300
CAATAATTAA AAAAATATGA ATTAATAGCA AAGCAGTGTG GACCATATAT          350
ACAAAAATCT ATAACAACAA CAAGGTGCAG AGCATTATTC CAACTAAGAT          400
CGAAGTTGTG ATACTGTCAT AATAAAAATG ACACATATTT TGACAACATA          450
AAAAATAAAT AACCATAAAA TATATCATAG AAAAATGAAT ATATTAGAAC          500
AGCTCACTCC AATATTAAAA GAGAGAAAAA AAATATTTTC CCACCACAAT          550
GCCATAATCC TTGAGCTTAG CTATTTATAA GTAAAAAAAA TGTTTTCTTG          600
GATAAATAGA AAAAGAAATA ATAATTAAAC ATAACCAATC ACTTCACAAA          650
TAAGAGTGTA TT <400>5                                            662
```

The level of homology between the potato and a tomato sequence is as follows:

```
Tomato:   307 ATTTATTTTTAGGAAAAATTATCTAAATACACATCTTATTTTACCATATACTCTAAAAAT 248
              | |||| |||||||||||||||  |||||||||| |||| | ||  |||| |||||||| |
Potato:  1914 AATTATATTTAGGAAAAATTACATAAATACACAACTTAATATATTATATTCTCTAAAATT 1973

          247 TCC 245   <400>6
              |||
         1974 TCC 1976   <400>7
```

This *Ds* line also exhibits a disease mimic phenotype (as does UQ406), indicating that the patatin gene may be involved in disease resistance and/or may act as an anti-oxidant in plant cells.

Homology is determined betwene UQ12 and a partial sequence encoding Ca2+ ATPase:

```
Bestfit of UQ12D73 and Ca2+ ATPase
.         .         .         .         .
     914 TTATACATTTCTGTTTGTATAAAGTGAAAGAGGAGAAGCAGAGAGTGGCG 865
         ||||| |||| | ||||||||||||||||||  || |  |||||| |||
    1015 TTATATATTTGTATTTGTATAAAGTGAAAGAGACGATG..GAGAGTAGCG 1062
                   .         .         .         .         .
```

```
 864 AGCGAGTTCCAGGAAGAGAAAAGAATGTCAATATGTTTTCTACGGATTAG 815
     ||||||  |   |  |||||     |||  |      |   | | |      |||||
1063 AGCGAGATTAAAAAAGAGTGGCGAACG.....AGATATGCCGTAAATTAG 1107
               .          .          .          .          .
 814 AATTAAATGAAACTGTAGCTATATTATTTATTTTTAAATTAATAATTTGC 765
     ||||||||||||||||    ||||  |||||||||  ||    |   | ||||
1108 AATTAAATGAAACTGTCATTATAACATTTATTTTGAATAAATAATTTTGA 1157
               .          .          .          .
 764 TATAATGCACAAATTTCCTTTAAAACGAAAAAAGTATTTGATAATGT 718
     ||||||  |||||  ||||   ||||||  |  ||   |       ||||||||
1158 TATAATACACAATTTTC..TTAAAAAGCAACGA......GATAATGT 1196
```

EXAMPLE 6

UQ11 mutant tomato plant

A mutant tomato plant designed UQ11, was subject to characterization. The UQ11 *Ds* insertion resulted from transposition of the *Ds* back into the T-DNA, but it is slightly closer to the right border and in the opposite orientation (Figure 13). Figure 12 shows the DNA sequence upstream of the UQ11 *Ds* insertion. Nucleotide 1 is the first nucleotide upstream of the *Ds* (and the active *nos:BAR* gene). The sequence for nucleotides 1 to 295 is T-DNA sequence corresponding to the right border of tomato transformant 1561E (5), the starting position of the *Ds* before lodging in the *Dem* locus. This is nucleotide sequence <400>10. Nucleotides 296 to 886 (in italics) [<400>11] correspond to tomato genomic DNA flanking the T-DNA insertion in 1561E. Note the *Bam*HI/*Bcl*I fusion sequence (TGATCC) and the *Hpa*I site (GTTAAC), both in bold in the Figure 12, immediately upstream of the insertion site (see Figure 1). The putative PMGSs of UQ11 reside in the right border of the T-DNA (nucleotide 1 to 295), and/or the flanking tomato DNA (nucleotide 296 to 886). Another PMGS may also be located further upstream.

EXAMPLE 7

PMGS in tomato mutant UQ14

A *Ds* insertion mutant, UQ14, resulted in *nos:BAR* expression. The transposon had, therefore, inserted proximal to a PMGS. The nucleotide sequences comprising PMGSs are represented in

<400>12 and <400>13.

A series of comparisons between <400>12 and other genes or nucleotide sequences was conducted:

(1) Homology between PMGS-UQ14 sequence [<400>14] upstream of Ds insertion and the 3' untranslated region of a potato sucrose synthase (susi) gene, Acc. no. AF067860 (70% homologous over about 200 bp):

```
                 .         .         .         .         .
PMGS-UQ14      40 TATGTTGCTCAAATCCTTCAAAAATCTCGACAGATGCATG.........G 80
                  |||||||||||||  |||||||||| || |||| ||| ||
Potato susi  7549 TATGTTGCTCAAACACTTCAAAAATGTCCACAGGTGCGTGTCGGATACTC 7598
                  .         .         .         .         .
PMGS-UQ14      81 CACCCGGTAGTGCATTTTTTTGAATGAGCTGGATACGAGTGCAATAATAT 130
                  ||    |||||| ||||   ||  ||     ||||  |||   |   |||
Potato susi  7599 CAAAAAGTAGTGTATTTAGGTGTGTG....TGATATTAGT...AGTGTAT 7641
                  .         .         .         .         .
PMGS-UQ14     131 ATTTGGGAAGTTTGAGCAAAATAGACCTGAAATTACTTTTAGCTTTTCTT 180
                  |||| ||  || || | | | |||   || | |||  | |   |  | ||
Potato susi  7642 ATTTAGG.TGTGTGTGGATAGTAG...TGTATTTAGATGTGTGTGATATT 7687
                  .         .         .         .         .
PMGS-UQ14     181 TTTTAAAG.............GAATCGGATATGGGTACAATAATATTTT 216
                  |   ||||              ||||  |||| |||| |   || | |||
Potato susi  7688 TCAAAAAGTTGTGTATTTTGGAGAATTTGATACGGGTGCGGCAACAATTT 7737
                  .         .
PMGS-UQ14     217 TGAAGAGTC.TGAGCAACATAG 237
                  |||||||||  |||||| ||||
Potato susi  7738 TGAAGAGTCAGGAGCAAAATAG 7759
```

(2) Homology between Region 1 of PMGS-UQ14 sequence (upstream of Ds insertion) and 3′ untranslated regions of potato sucrose synthase and two other genes, namely:

a) 3' untranslated region of a potato sucrose synthase (susi) gene, Acc. no. AF067860 (83% homologous over 41 bp),

b) 3' untranslated region of a potato lactate dehydrogenase (LDH) gene (85% homologous over about 41 bp), and

c) intron 2 of the tomato phytochrome B1 (PHYB1) gene, Acc. no LEAJ2281

(95% homologous over 22 bp).

```
a)

PMGS-UQ14      40 TATGTTGCTCAAATCCTTCAAAAATCTCGACAGATGCATGGC 81
                  |||||||||||||  |||||||||| || |||| ||| || |
Potato susi  7549 TATGTTGCTCAAACACTTCAAAAATGTCCACAGGTGCGTGTC 7590

b)

PMGS-UQ14      39 CTATGTTGCTCAAATCCTTCAAAAATCTCGACAGATGCATG 79
                  |||||||||||||||||||||||||| ||    ||||| ||
Potato LDH    704 CTATGTTGCTCAAATCCTTCAAAAATGTCATTGGATGCGTG 744

c)

PMGS-UQ14           40 atgttgctcaaatccttcaaaaa 62
                       |||||||||||||||| ||||||
Tomato PHYB1      6781 atgttgctcaaatcctccaaaaa 6803
```

(3) Homology between Region 2 of PMGS-UQ14 sequence (upstream of Ds insertion) and untranslated regions of five other genes, namely:

a) 3' untranslated region of a potato sucrose synthase (susi) gene, Acc. no. AF067860 (74% homologous over 38 bp),

b) 3' untranslated region of a potato lactate dehydrogenase (LDH) gene (75% homologous over about 47 bp),

c) intron 1 of a potato cytosolic pyruvate kinase gene, Acc. no STCPKIN1 (71% homologous over 58 bp),

d) genomic sequence downstream of a *Brassica napus* 1.7S seed storage protein napin *(napA),* Acc. no. BNNAPA (71% homologous over 58 bp), and

e) 3' untranslated region of a tomato chorismate synthase 2 precursor (CSP) gene, Acc no. LECHOSYNB (95% homologous over about 23 bp).

```
a)

PMGS-UQ14      189 GAATCGGATATGGGTACAATAATATTTTTGAAGAGTCTG 227
```

```
                    ||||  ||||  ||||  |    ||  |  ||||||||||||  |
Potato susi   7710 GAATTTGATACGGGTGCGGCAACAATTTTGAAGAGTCAG 7748
```

b)

```
PMGS-UQ14      238 TCTATGTTGCTCAGACTCTTCAAAAATATTATTGTACCCATATCCGAT 191
                   ||||||||||||||  |  ||||||||||  |  ||||  |   |  |  |  |||
Potato LDH     703 TCTATGTTGCTCAAATCCTTCAAAAATGTCATTGGATGCGTGTTGGAT 750
```

c)

```
PMGS-UQ14 179  TTTTTTAAAGGAATCGGATATGGGTACAATAATATTTTTGAAGAGTCTGAGCAACATAG 237
               ||  |||    |  |||  ||||  |  ||||  |  ||  |  |||||  ||||  ||||||||||
Potato CPK 951 TTCTTTTTGAGGATCCGATACGAGTACGACAACAATTTTGGGGAGTTCGAGCAACATAG 1009
```

d)

```
PMGS-UQ14 227 CAGACTCTTCAAAAATATTATTGTACCCATATCCGATTCCTTTAAAAAAGAAAAGCTAA 169
              |||  ||  |  ||||||  |||  |||  |    ||  |    |||   ||  ||||||||||||  |||
napA     2902 CAGTCTGTACAAAAAAATTTTTGAATAAATTTAACATTATTTCAAAAAAGAAAAGGTAA 2960
```

e)

```
PMGS-UQ14    202  acaataatatttttgaagagtct 224
                  ||||  ||||||||||||||||||
Tomato CSP 1630  acaacaatatttttgaagagtct 1652
```

EXAMPLE 8

Tagging additional genes involved in carbon metabolism

As the above indicates, selecting for transposition of a methylated *Ds* from the *Dem* locus and for expression of the *nos:BAR* gene (i.e.: demethylation of the *Ds*) efficiently identifies *Ds* insertions into regions homologous to DNA sequences of known function, as opposed to so-called "junk DNA". In all of the above cases, the *Ds* insertion is in the vicinity of a region homologous to DNA sequence of known function.

The five lines carrying active *nos:BAR* genes associated with regions homologous to DNA sequences of known function are:

- *Ds* insertion in UQ406 - associated with the promoter of an α-amylase gene (Example 3, above);
- *Ds* insertion in UQ12 - associated with a putative patatin gene (Example 5);
- *Ds* insertion in UQ11 - associated with the Right Border of the *Agrobacterium* T-DNA 1516E (refer to Figures 12 and 13 and Example 6). This was the T-DNA carrying the *Ds* that was initially transformed into tomato. In other words, the *Ds* transposed from the *Dem* locus back into the T-DNA;
- *Ds* insertion in UQ14 - associated with or closely linked to a putative sucrose synthase gene (see Example 7); and
- *Ds* insertion in UQ13 - associated with or closely linked to a putative UDP-glucose-pyrophosphorylase gene and/or expansin, genes potentially involved in starch biosynthesis.

In four of these instances, the *Ds* is associated with DNA sequences related to carbon (C) metabolism (α-amylase, patatin, sucrose synthase and UDP-glucose-pyrophosphorylase). Since several of these lines are characterised by a disease mimic phenotype, this implies that a patatin gene and a sucrose synthase gene (and probably other C metabolism genes) are involved in disease resistance. These data also indicate that many metabolism genes and many so called house-keeping genes contain demethylation sequences or sequences which prevent or reduce methylation.

The portions of the nucleotide sequence downstream of the *nos:BAR* insertion in UQ13 were compared with the nucleotide sequences for tomato expansin 2 ADP-glucose pyrophosphorylase and Ca^{2+} ATPase. The Bestfit analysis is shown below:

```
Bestfit of UQ13D73 and Expansin 2

                .         .         .         .         .
 510 GGTCGTTTGGCATAAAAATACATAATGCAGGGATTATTAACGTATAGATT 559
     | ||||  || | ||| ||  ||| | ||||              || |
4233 GATCGTACGGTACAAAGATCAATACTTCAGG.............GAGT 4267
                .         .         .         .         .
```

```
 560 AGTAATACATAGATTAGTAATGCATGGATTAGTTTTTATCAAGTGTTTGA 609
     ||||||||||   || ||||||||  ||||| ||||||||||||||||||
4268 AGTAATACATTTTTTGGTAATGCAGAGATTA.TTTTTATCAAGTGTTTGG 4316
           .         .         .         .         .
 610 TTCATTGTTTCCTACTTAATCTTATGTTTAGTTTAAAACTCTAGAAAAAT 659
     ||||||||||  ||| |||| || ||| | |||||||  |    ||||||
4317 TTCATTGTTT.TTACCTAATTTTGTGTGTGGTTTAAAGTTTACAAAAAAT 4365
           .         .         .         .         .
 660 A..TATTTCCTATTATACCTTTGAGTTATTGTGAGAATTTGTATTTCATT 707
     |  | |||||  |||||||  |  |||||||  |||||  |||  ||||||||
4366 AATTCTTTCCAATTATACGCTAAAGTTATTATGAGATTTTATATTTCATG 4415
           .         .         .         .         .
 708 TAACT.AGTCAAGTTAAATNCNAATTTATATATATATATATATTATTA 756
     |||  |  |||||   ||  : :|||| |   |||    |||  | |||| |
4416 TAATTGGGTCAA...AATAGATAATTGACCGATAATATTATTTTTTATAA 4462

 757 ATTTT 761
       |||
4463 CATTT 4467
```

Bestfit UQ13D73 and Tomato ADP-glucose pyrophosphorylase

```
542 ATTATTAACGTATAGATTAGTAATACATAGATTAGTAATGCATGGATTAG 591
    ||||||    |  |||||| |||| ||| || || |||||   || |||
2035 ATTATTGGTATCGAGATTAATAATGCATTGACTAATAATGTCGGGTTTAT 2084
           .         .
 592 TTTTTATCAAGTGTTTGATTCATT 615
     ||||||||||||  |||||  | |
2085 TTTTTATCAAGTGAATGATTGAGT 2108
```

EXAMPLE 9

A rapid bioassay for identification of tomato DNA sequences capable of alleviating transgene silencing in a heterologous plant species

An efficient transformation system has been developed for sugarcane, based on particle bombardment of embryogenic alleles, followed by plant regeneration (17). The bioassay is useful for identifying tomato sequences which prevent transgene silencing and employs the plant expression vector pZorz. This plasmid carries a firefly luciferase reporter gene *(luc)*, under the

control of the *Osa* promoter (12). After bombardment of embyrogenic callus of sugar cane, the luciferase gene is expressed, as determined by protein assay or observed by visualisation of the chemiluminescence of the luciferase enzyme. However, in normal sugarcane, it becomes completely silenced upon regeneration. The silencing appears to be correlated with methylation of the transgene. This phenomenon was used to test the effect of putative PMGSs, as follows.

Expression vector pZorz (12) was digested with *Hin*dIII and an approximately 20bp oligonucleotide, containing a *Not*I restriction site and overhanging ends complementary to the *Hin*dIII site, was ligated into the *Hin*dIII site at position 1 of the pZorz backbone just upstream of the *Osa* promoter. The ligation results in the loss of the *Hin*dIII site. The new plasmid was designated pUQ511 (Figure 7).

Plasmid pUQ511 was then partially digested with *Eco*RI, to isolate the full-length linearised plasmid. This plasmid was ligated with another approximately 20bp oligonucleotide, containing a *Sma*I restriction site and overhanging ends complementary to the *Eco*RI site. This ligation results in the loss of the *Eco*RI site. Religated plasmids containing the new *Sma*I site at position 1370 of the pZorz backbone, just downstream of the nos terminator, were selected by PCR and this new plasmid was designated pUQ505.

Plasmid pUQ505 or pUQ511 were used as the starting vectors for constructing expression vectors containing putative PMGSs for bioassay. Tomato sequences flanking the reactivated *nos:BAR* insertions of UQ406, UQ11 and UQ14 were inserted into pUQ505 at the *Not*I site and into pUQ511 at either the *Not*I site or the *Eco*RI site or both. For example, pUQ505 was partially digested with *Not*I and the putative 886 bp-PMGS from UQ11, as shown in <400>9, was ligated into the new *Not*I site (formed as described above), in both orientations, to generate pUQ527 and pUQ5211 (Figure 7).

These modified pZorz expression vectors were used with a transformation marker to transform sugarcane, in order to test whether the PMSGs are capable of alleviating silencing of the *luc* gene. Smaller fragments are then generated by deletion analysis and subcloned into expression vectors, to more accurately define the effective sequences.

Tomato sequences flanking reactivated *nos:BAR* in UQ406, UQ11, UQ12, UQ13 and UQ14 are also introduced next to a *nos:BAR, nos:LUC* or *nos:GUS* recombinant gene in another plasmid vector. These modified recombinant *BAR, LUC* and *GUS* genes are inserted into binary vectors (4) for transformation into *Arabidopsis thaliana* (18) to test the ability to prevent silencing of the *nos:BAR* gene in *Arabidopsis.*

EXAMPLE 10

Analysis of sequences responsible for reactivating *nos:BAR* expression

The borders of DNA elements that prevent transgene silencing are initially defined by deletion analysis of clones that yield positive results in the bioassays. The smallest active clone for each chromosomal region is then sequenced and characterised in detail. Sequences from independent *Ds* insertions are compared for homologous DNA elements.

EXAMPLE 11

Modification of plant photosynthetic architecture by *Ds* transposon tagging

As stated in Example 2, UQ406 carries a single transposed *Ds* element (without the transposase gene which has segregated away) and is characterised by showing an improved seedling growth, and a disease mimic or premature senescence phenotype on mature leaves. UQ406 also possesses an active *nos:BAR* gene indicating that the insertion caused two phenotypes: namely premature senescence and reactivation of the *nos:BAR* gene inside the *Ds* element.

Surprisingly, DNA sequence analysis shows that the *Ds* insertion in UQ406 is located only about 3 kb upstream from the ATG of the *Dem* (Defective embryo and meristems) gene which has been cloned by tagging with *Ds* (Example 4). In fact, only about 700 bp of DNA separates the putative α-amylase STOP codon and the *Dem* ATG codon (Figure 8). This region presumably contains the promoter of *Dem* locus and its nucleotide sequence is shown in <400>8. The *Dem* gene is required for correct patterning in all of the major sites of differentiation, namely in the embryo, meristems, and organ primordia. The function of *Dem* was determined by STD, somatic tagging of *Dem.* Figure 8 provides a diagrammatic representation of the STD genotype. Mutant *dem+7*

is a stable frameshift mutant of *Dem*, TPase represents a T-DNA 3 centiMorgans (cM) from *Dem*, carrying the *Ac* transposase and a GUS reporter gene. The transposase is required for *Ds* transposition. The location of stably inherited (shaded) and somatic (open) *Ds* insertions in the *Dem* locus and an upstream α-amylase gene is shown in Figure 8b. The α-amylase gene is in the same orientation as *Dem*. Coding sequences plus introns are shown as boxes and the dark section of the *Dem* locus represents an intron. All of the 8 somatic insertions shown were associated with palisade deficient sectors. The genomic region represented in Figure 8b has been sequenced (see Figure 5; please note that the intron in the *Dem* locus is not included in this sequence). As shown in Figure 8c mutant *dem* sectors lack palisade cells (p, palisade cells, s, spongy mesophyll, g, wild-type dark green sectors, and lg, mutant light green sectors). The inventors have shown, therefore, by somatically tagging *Dem* with *Ds*, that the gene is involved in cell growth during plant differentiation (Figures 8 and 9).

As stated above, the sequence flanking the active *nos:BAR* genes are referred to herein as "Phenotype modulating genetic sequences" or "PMGSs".

Another genotype has been produced for the somatic tagging of the *Dem* gene, further demonstrating the involvement of the *Dem* gene in cell growth. The genetic derivation of somatically-tagged *Dem* is shown in Figure 10. Besides palisade-less sectors (Figure 8), a new phenotypic class is characterized by multicellular palisade tissue. In the wild-type tomato, the palisade tissue is composed of a single long columnar palisade cell. In the new mutant sectors, which look wild-type to the naked eye, the long columnar cell is replaced by several smaller cells packed on top of one another. This is shown in Figure 11. Each mutant sector arises from an independent insertion of *Ds* in the *Dem* gene. The different classes of mutant sectors apparently result from different classes of mutations in the *Dem* gene and also indicates that *Dem* is involved in cell division as well as cell growth, expansion and/or division.

Somatically-tagged *Dem* plants are crossed to a stable null mutant of *Dem* and progeny are screened to identify stable mutant lines with genetically-modified palisade tissue. Lines exhibiting beneficial characteristics, such as increased levels of photosynthetic activity, can then be selected. Lines resulting from other *Dem* alleles and exhibiting other beneficial

modifications, for example altered developmental architecture such as modified cell, tissue or organ growth rate, shape or form, may also be identified.

EXAMPLE 12

Transposon tagging of α-amylase gene

The inventors have used the transposon tagging system described in Example 4 to introduce a transposon into the α-amylase gene. One mutant line obtained was UQ406.

The DNA from 651 bp of the upstream of the UQ406 insertion down to the end of the *Dem* coding sequence has been sequenced (Figure 5). The close proximity of the α-amylase gene to the *Dem* cell growth gene indicates that these genes may play a key role in cell growth, expansion and/or division and differentiation. Several heterozygous insertion mutants are identified in the α-amylase coding sequence and these are selfed to produce plants homozygous for the *Ds* insertion in the α-amylase coding sequence. If these have a similar or more or less severe phenotype to the plants homozygous for the stable *Dem* insertion mutant, then this will indicate that indeed this cloned α-amylase gene plays a key role in cell growth, expansion and/or division and, therefore, the shape and growth of plants.

A tomato chromosomal region spanning these genes is cloned into an *Agrobacterium* binary vector (19) to produce plasmid pUQ113, and this plasmid is introduced into *Arabidopsis* by method of Bechtold and Bouchez (18) to modify the cell shape and growth of this other plant species. A T-DNA insertion mutant in the *Dem* gene is identified in *Arabidopsis* and this mutant is also transformed with pUQ113 to modify the cell shape and growth of *Arabidopsis*.

Recombinant combinations of α-amylase and/or *Dem* genes are transformed into a range of plant species to modify the cell shape and growth of the species.

EXAMPLE 13

Genetic engineering of disease resistance and senescence based on modification of expression of α-amylase

Ds insertion mutant UQ406 is characterized by a lesion mimic phenotype. The mutant phenotype is evident in mature leaves, but not in young leaves or any other tissue. No pathogens are found in leaf tissue displaying this phenotype. The dominant nature of the UQ406 phenotype and the location of the *Ds* in the α-amylase promoter suggest that over-, under or constitutive expression of the gene may be responsible for activating a disease resistance response and/or senescence in mature leaves. These data and the very close proximity of the α-amylase and *Dem* genes are also consistent with co-ordinate regulation of these genes in differentiating tissue. Induction of disease resistance and plant senescence, to produce desirable outcomes in crops and plant products, may, therefore, be able to be controlled by modification of α-amylase expression.

An early event in the disease response of a challenged plant is a major respiratory burst, often referred to as an oxidative burst due to an increase in oxygen consumption. This burst of oxygen consumption is due to the production of hydrogen peroxide (H_2O_2) linked to a surge in hexose monophosphate shunt activity (20). This activity results from the activation of a membrane-bound NADPH oxidase system which catalyses the single electron reduction of oxygen to form superoxide (HO_2/O_2^-), using NADPH as the reductant (20). Spontaneous dismutation of HO_2/O_2^- then yields H_2O_2. Consumption of glucose *via* the hexose monophosphate shunt (alternatively known as the cytosolic oxidative pentose phosphate pathway) regenerates the NADPH consumed by the NADPH oxidase system. It is, therefore, entirely conceivable that an α-amylase is responsible for supplying sugars required by the pentose phosphate pathway, and perhaps for the primary activation of the signal transduction pathway that leads to disease resistance in plants.

Following the oxidative burst, disease resistance is manifested in localised plant cell death called the hypersensitive response (HR), in the vicinity of the pathogen. The HR may then induce a form of long-lasting, broad spectrum, systemic and commercially important resistance known as systemic acquired resistance (SAR). The compounds, salicylic acid, jasmonic acid and their methyl derivatives as well as a group of proteins known as pathogenesis related (PR) proteins are used as indicators of the induction of SAR (23).

Increased levels of sugars have been related to heightened resistance especially to biotrophic pathogens (21). When invertase (the enzyme responsible for the breakdown of sucrose to

glucose and fructose) is overexpressed in transgenic tobacco, systemic acquired resistance is induced (22).

The α-amylase coding sequence is inserted behind an inducible promoter and transformed into plants to confer a inducible disease resistance in plants. Similarly, the α-amylase coding sequence is inserted behind an inducible promoter and transformed into plants to confer inducible senescence in plants for the production of desirable products or traits.

When a disease resistance response is invoked in one part of a plant, a general and systemic acquired enhancement in disease resistance is conferred on all tissues of such a plant (21). Tomato line UQ406 is tested for enhanced resistance to a wide range of pathogens to test this hypothesis.

EXAMPLE 14

Modifications of carbon metabolism

As stated in Examples 7 and 8, in four of the five lines carrying active demethylated *nos:BAR* genes, the *Ds* has inserted into or near sequences homologous with carbon metabolism gene. These results indicated that many C metabolism genes have *cis*-acting sequences which prevent methylation and concomitant gene silencing. Demethylation sequences are inserted next to recombinant C metabolism genes to enhance their expression and modify C metabolism in beneficial ways; such as up-regulation of the sucrose phosphate synthase gene in sugar cane, to yield higher concentrations of sugar in beneficially-modified plants.

EXAMPLE 15

Cloning of downstream genes associated with plant cell apoptosis caused by *Ds* insertion

A cDNA library is made from tomato leaf tissue showing the disease mimic (apoptosis) phenotype caused by *Ds* insertion in UQ406. This library is screened differentially with two probes, one being cDNA from normal tissue and the other being cDNA made from leaf tissue

showing the disease mimic phenotype caused by *Ds* insertion. This procedures identifies genes specifically-induced during plant cell death. These apoptosis-associated genes are then sequenced, and compared with other genes present in the DNA databases. The proteins encoded by these genes are expressed *in vitro* and tested for their ability to kill plant cells.

EXAMPLE 16

Analysis of *Dem* and its product DEM

1. DEM in differentiating cells

A truncated version of DEM protein is expressed *in vitro* from an *E. coli* pET expression vector. Polyclonal antibody is raised against this truncated DEM protein in mice. In Western blots, the polyclonal antibody specifically recognizes a protein of the predicted size of the DEM protein in shoot meristem tissue. This antibody is employed in immunolocalization experiments. Tomato shoot and root meristematic regions and leaf primordia are processed for electron microscopy and immunolocalization of DEM. The technique employs gentle aldehyde crosslinking of the tissues and infusion with saturated buffered sucrose before freezing the samples in liquid nitrogen. Mounted blocks are then thin sectioned at low temperature at low temperature and immunolabelled with fluorescent or electron dense markers suitable for electron microscopy, a room temperature. An advantage of this methodology is the excellent ultrastructural preservation, combined with the retention of antigenicity which allow for meaningful antigen-antibody localisation of proteins. Results show that the polyclonal antibody detects an antigen in the outer cell layer of shoot meristem tissue.

2. Cell walls

Standard analytical techniques are used to analyse and compare cell wall compositions of mutant *dem* and wild-type tissue.

3. Function of the *DEM* homologue *(YNV212N)* in yeast

The mature N-terminal sequence of the DEM protein, MGANHS conforms to the consensus sequence for N-myristoylation. This consensus sequence appears to be missing from the predicted YNV212W protein based on genomic sequence. A full length yeast YNV212W cDNA is cloned and sequenced, and gene disruption techniques are used to introduce frameshift mutations at several locations along the YNV212W coding sequence. By generating frameshift mutations at several points along the gene, mutant alleles of YNV212W are created. The resultant mutants are observed for modified growth and morphology. There are no other genes in yeast that are homologous to YNV212W. YNV212W cDNA is cloned into an inducible expression vector for yeast, and yeast strains overexpressing YNV212W are observed for changes in growth and morphology.

4. Identification of wild-type and mutated *Arabidopsis* genes that are homologous to *Dem*, and observation of insertion mutants for altered morphology

BLAST searches (25) using the tomato *Dem* nucleotide sequence has identified three separate homologous sequences in *Arabidopsis* (accession numbers AB020746, AC000103 and ATTS5958). The level of homology to the tomato gene ranges from 56 to 68% on the nucleotide level over 350 to 800 bp and indicates that there may be several genes related to *Dem* in plants. Full length *Arabidopsis* cDNAs homologous to the tomato *Dem* cDNA are cloned and sequenced. Antisense constructs under control of the cauliflower mosaic virus 35S promoter are made and transformed into *Arabidopsis* and the resulting transformants are observed for morphological abnormalities. Insertion mutants in *Dem* homologues are identified from the *dSpm* and T-DNA tagged lines of *Arabidopsis*. Insertion mutants are screened for modified morphology.

5. Identification and characterization of additional stable Ds insertions in the vicinity of *Dem* and screening for mutants with modified photosynthetic architecture

Up to 2,000 STD progeny lacking the *Ac* transposase (detected by absence of the GUS reporter gene) are screened by PCR for *Ds* insertions in the region of *Dem*. DNA is extracted from bulk leaf samples of 50 plants and used as template in 8 PCRs. All 8 reactions include oligonucleotide

primers facing away from both sides of *Ds*. The 8 separate PCRs vary according to the oligonucleotide primer used to anneal to the tomato genomic sequence. These 8 primers are evenly distributed, 1kb apart along the tomato sequence. Amplification of a fragment indicates a *Ds* insertion in the vicinity of *Dem*. When a fragment is amplified from a DNA sample, the PCR product is authenticated by a nested PCR. Subsequently, the individual plant carrying the *Ds* insertion in the vicinity of *Dem* is identified by the appropriate PCR assay, using intact leaf tissue as template. Plants homozygous for new stable *Ds* insertions in the vicinity of the *Dem* locus are morphologically characterized, both in terms of meristem structure and organization of photosynthetic tissue. New lines showing modified morphology are crossed to a line expressing *Ac* transposase. Instability of the phenotype in the presence of transposase will confirm that a *Ds* element is responsible for the modified morphology.

The progeny from STD plants are also screened directly for stable mutants in the photosynthetic architecture of leaves. The screen involves hand-sectioning the tissue, then toluidine blue staining followed by light microscopy. This method results in the isolation of genetically-stable multicellular palisade mutants. Mutants are crossed to a line expressing *Ac* transposase to determine if the mutation is due to a *Ds* insertion. If the phenotype shows instability in the presence of transposase, the corresponding gene is cloned and characterized.

6. Antisense *Dem* constructs for transformation into tomato

Antisense constructs involving the tomato *Dem* coding sequence are produced and transformed into tomato with the aim of producing a large number of tomato lines that vary in DEM function. The antisense constructs are made under the control of the 35S promoter. Thirty transformants are produced and observed for modified growth and morphology. Microscopy is used to characterize the organization of photosynthetic tissue in these antisense lines.

EXAMPLE 17

Analysis of PMGSs

The PMGSs in mutant lines such as UQ11, 12, 13 and 14 and 406 are analysed in a number of

ways. In one analysis, the right border (RB) and or flanking DNA in a *Ds* containing line in which *nos:BAR* is expressed is used to screen for stabilized expression of transgenes. For convenience, transgenes encode a reporter molecule capable of providing an identifiable signal. Examples of such reporter transgenes include antibiotic resistance.

In addition, genetic constructs comprising nucleotide sequences carrying PMGSs flanking *nos:BAR* are inserted next or otherwise proximal to selectable transformation marker genes such as *BAR* or *NPT* and the resulting plasmids are used in transformation experiments to enhance the transformation efficiency of plant species such as wheat and sugar cane.

EXAMPLE 18

Therapeutic application of PMGSs

Latent viruses such as HIV-1 may employ mechanisms such as methylation to remain inactive until de-methylation occurs. The PMGSs of the present invention may be used to de-methylate and activate latent viruses such as HIV-1 so that such viruses can then be destroyed or inactivated by chemical or biological therapeutic agents.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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